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A Thesis for the Degree of Master of Science

**The Role of Different Atmospheric Pressure  
Plasma Treatments for Inactivation of Bacterial  
Biofilms on Stainless Steel**

스테인리스에 형성된 바이오필름 병원성 미생물에  
대한 플라즈마 처리 방식에 따른 살균 효과

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## List of Abbreviation

EPS	Extracellular polymeric substances
QS	Quorum sensing
RTE	Ready-to-eat
PAA	Peracetic acids
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
<i>LD</i>	<i>Longissimus dorsi</i>
DBD	Dielectric barrier discharge
PTW	Plasma treated water
APP	Atmospheric pressure plasma
PTS	Plasma treated solution
CLSM	Confocal laser scanning microscopy
SEM	Scanning electron microscope
ORP	Oxidation reduction potential
DW	Distilled water
TSB	Tryptic soy broth
TSA	Tryptic soy agar

# **I. General introduction**

The foodborne disease is explained as the illnesses with which people are infected by the foods they eat. Contamination of the food by microorganisms at any stage, from production to consumption, which eventually causes the foodborne disease (Dorne et al., 2009). These diseases considered to be critical problems that can lead to threat international public health safety and economic development. To improve the food safety, food industries have used a various food processing methods such as pasteurization, canning, refrigeration, drying, freezing, curing, food preservatives, and food irradiation.

Despite the many efforts, foodborne disease has occurred continuously and seriously (Nyachuba et al., 2010). It was estimated that 76 million cases of foodborne diseases occur each year, causing 325,000 hospital cases and 5,000 deaths in the United States according to the Centers for Disease Control and Prevention (CDC) (Schlisselberg and Yaron, 2013). Especially, 349 cases of foodborne disease occur in 2014, causing 7,466 hospital cases in Korea (Seong et al., 2016).

The foods most commonly incriminated include meat and dairy products, eggs, fish, and raw vegetables, especially, animal origin foods are a rich nutrient matrix that provides a suitable conditions for proliferation of microorganisms (Aymerich et al., 2008). Especially, pathogens such as *Listeria monocytogenes*, *Salmonella* spp., and

*Escherichia coli* O157:H7 can grow and cause foodborne disease (Buchanan and Whiting, 1986).

On the other hand, it has become increasingly clear that bacteria as referred previously grow predominantly as biofilms on surfaces, in most of their habitats, rather than in planktonic cells in meat industry (Lindsay and von Holy, 2006). Biofilms are responsible for over 65% of all microbial diseases (Potera, 1999). It has been observed that the biofilm is increased the antimicrobial resistance compared with the planktonic cells. Biofilms formed by pathogenic and spoilage bacteria may create a cross-contamination to product, leading to serious hygienic problems and also economic losses (Sofos and Geornaras, 2010). Therefore, efficient inactivation methods must be applied to preserve meat safety.

Various disinfection methods have been used to protect biofilm development in food industry. The conventionally used sanitizer in food processing was sodium hypochlorite (NaOCl). NaOCl has advantages to be cheap and to be effective in a broad microbial spectrum. However, the formation of disinfection by-products such as trihalomethanes and haloacetic acids has been considered harmful to health (Haute et al., 2015). Peracetic acid also has been used for disinfectants because it is effective in low concentration, non-toxic, and penetrates biofilms. However, it can be corrosive and is unstable. Hydrogen peroxide can weaken biofilms and supports detachment of cells to surface but it needs high concentration and it can be corrosive to the surface (Wirtanen and Salo, 2003). As for the increase in concerns about disadvantages of

chemical sanitizers, the alternative disinfection methods have been studied.

Recently, bacteriophage has been proposed as a biofilm control method. Several phages produce enzymes that degrade the extracellular polymeric substances (EPS) and the biofilm matrix (Fu et al., 2010). Besides, the enzyme-based detergents such as detachment-promoting agents (DW-PAs) of polysaccharide depolymerases, esterases, and dispersin B might break the EPS and disinfectants could affect the biofilm (Xavier et al., 2005).

Among them, the plasma is on the rise to use for one of the disinfection methods of biofilm. Plasma consists of electrons, photons, positive and negative ions, neutral atoms, free radicals, UV photons and reactive species. These chemical species and UV light have been widely reported to play a major role in disinfection. Several plasma systems have been developed. Among them, cold atmospheric pressure plasma has attracted attention as the non-thermal plasma at atmospheric pressure and it makes the disinfection process practical and cost effective (Kim et al., 2013). Bacterial inactivation effect of plasma has been studied using various types. There were studies that have been investigated on disinfection by direct plasma such as *Pantoea agglomerans* on bell peppers (Vleugels et al., 2005). Laurita et al. (2015) reported that there was bacterial inactivation effect by post-treatment duration referring to trap the chemical species after plasma discharge was switched off in closed environment. In recent studies, it has been reported that the plasma

treated water can also be an efficient disinfection solution due to chemical species produced by plasma treatment (Zhang et al., 2013). However, the study related to inactivation effect to biofilm in food industry using plasma was limited.

The objective of this study was to investigate the inactivation effect of cold atmospheric pressure plasma against pathogenic bacteria in biofilms formed on stainless steel used in meat industry and to elucidate its inactivation mechanism.

## II. Literature review

### 1. Biofilm

#### 1.1. What is biofilm?

Biofilm is defined as the sessile community of microbes characterized by cells that are irreversibly adhered to biotic or abiotic surface and embedded in a matrix of EPS. Unlike planktonic cells, the bacteria forming biofilm exhibits transformed phenotypic and genotypic characteristics such as the production of EPS and the release of enzymes associated with biofilm formation due to gene expression. Microbes prefer to live as biofilm phase as they could be prevented from antimicrobial agents through EPS (Donlan and Costerton, 2002). Biofilm can be formed by various types of microorganisms such as pathogenic and spoilage microorganisms under appropriate environments (Nivens et al., 1995).

#### 1.2. Mechanism of biofilm formation

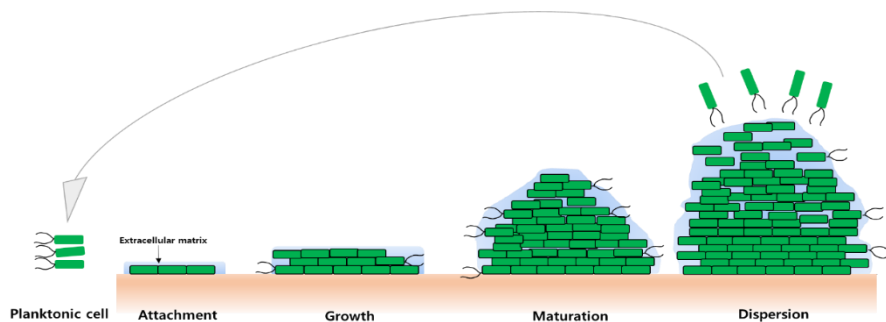


Fig. 1. Schematic representation of biofilm formation

Biofilm formation is commonly classified four stages (Fig. 1). The first stage begins with the bacterial attachment to surface, followed by the formation of microcolony, and biofilm maturation. The final stage of biofilm formation cycle is dispersion that the cells revert into their planktonic stage in order to contaminate other surface (Donlan and Costerton, 2002).

#### *Attachment*

In this step, cells are still reversible to surface. The interaction between cells and surfaces depends on many physical, chemical, and biological forces. The cell approaches to surface when exposed to nonspecific physiochemical forces such as Van der Waals forces, surface hydrophobicity, produce hydrophobicity, and electrostatic forces. Moreover, the planktonic bacteria's attachment to surface is influenced by their motility or the gravitational transportation (Bos et al., 1999). Karatan and Watnick (2009) reported that motile bacteria which has flagella motor senses the interaction with the surface. This phenomenon induces a signal that triggers the gene expression related to biofilm formation and inhibits the motility of bacteria.

#### *Microcolony formation*

Followed by the attachment, the cells begin to proliferate to microcolonies. Reversible attachment is replaced into irreversible microcolonies through EPS that forms the biofilm matrix. EPS consists of polysaccharides, nucleic acids, proteins, and lipids. These substances enable microcolonies to adhere to surfaces and to each other. And the

development of biofilm matrix also resulted from quorum sensing (QS) which regulates the cell-to-cell communication (Flemming and Wingender, 2010).

### *Maturation*

The process of biofilm maturation forms an organized structure – flat or mushroom shaped under control of several environmental signals such as temperature and nutrient availability. A period of 10 days or more is required in order to reach mature structure. Such structure stabilizes the three-dimensional biofilm matrix (Stoodley et al., 2002).

### *Dispersion*

The final stage of the biofilm formation is dispersion which turns the sessile cells into planktonic state to contaminate other surfaces and then to repeat the cycle. An alteration to nutrient starvation, oxygen depletion, and other stress conditions is considered as reasons for dispersion. These extreme circumstances allow bacteria to find a nutrient-rich environment and trigger the gene expression associated with dispersion (McDougald et al., 2011). Biofilm can be not only a source of contamination but also a defense protecting antimicrobial from inactivating the bacteria (Donlan and Costerton, 2002).



### **1.3. Biofilm-forming foodborne pathogens**

#### *Listeria monocytogenes*

The presence of *L. monocytogenes* in food processing is a critical consideration. *L. monocytogenes* can exist for long periods, being able to persist for month or years in location such as floor and drains after contaminating the facility (Olszewska et al., 2016). The ability of bacteria to replicate at low temperature and survive for long terms within the environment under adverse conditions has made *L. monocytogenes* a causative concern for the manufacturing and food processing industries (Alonso et al., 2014). Gamble and Muriana (2007) reported that *L. monocytogenes* is well known for its ability to form biofilm and to develop harborages on food plants, making its elimination more difficult. *L. monocytogenes* does not always form a strong biofilm or EPS network after attachment when in single culture, however, it has been observed to grow or survive in multispecies biofilms (Hassan et al., 2004). *L. monocytogenes* can form mixed-species biofilm with e.g., *Pseudomonas* and *Salmonella* (Olszewska et al., 2016).

#### *Escherichia coli* O157:H7

*E. coli* O157:H7 is known to form biofilm on various types of food contact surfaces such as glass, stainless steel, and plastic. *E. coli* O157:H7 biofilm formation is associated with the expression of curli and EPS. Bacteria curli mediates attachment to surfaces (Uhlich et al., 2006). *E. coli* O157:H7 is major pathogens that need to be controlled in fresh meat since it can attach and grow under nutrient starvation, if

temperature is optimal. *E. coli* O157:H7 can adhere to stainless steel and high density polyethylene food contact surfaces during cold storage (4°C) as well as at higher temperature (15°C). In addition, *E. coli* O157:H7 had a stronger strength of attachment on dry surfaces making its removal more difficult (Sofos and Geornaras, 2010).

#### *Salmonella spp.*

*Salmonella* spp. is a major causative factor of human salmonellosis which are transmitted by food. Numerous studies have shown that this bacteria is able to form biofilm on metal, glass, plastic, or rubber surfaces (Stepanović et al., 2003). Especially *Salmonella* can be isolated from poultry processing equipment in the slaughter and evisceration area (Helke and Wong, 1994). Jones and Bradshaw (1997) showed that *Salmonella* can attach and form biofilms on pipelines found in poultry processing plants.

#### *Bacillus spp.*

In dairy manufacturing plants, *Bacillus* is actively attached to surfaces and forms biofilm. It survives heat processing, increases on pipelines, and remains in the processing environment (Parker et al., 2001). The thermophilic *Bacillus* is difficult to eradicate from a manufacturing process because of the resistance of their spores to heat and chemicals and their ability to formation of biofilm. When the spores once adhered to a surface, the spores germinate and grow forming a biofilm. The biofilm is likely to contaminate the milk according to the release of planktonic cells from the biofilm (Scott et al., 2007).

*Pseudomonas spp.*

*Pseudomonas* is found in tendon slices and the growth of *Pseudomonas* formed biofilm on the surface of milk processing lines (Hood and Zottoloa, 1997; Maxcy, 1972). It forms amounts of EPS and has been shown to adhere and form biofilms on stainless steel surfaces. *Pseudomonas* coexist within biofilms with *Listeria*, *Salmonella* and other pathogens. (Barnes et al., 1999).

#### **1.4. Biofilm in animal origin food industry**

##### *Meat*

During the slaughter, a various sources including feces, hide, water, air, and handling can contaminate carcasses. Skinning and evisceration cause enormous dispersion of biofilms including shigatoxin *E. coli* (STEC) and *Salmonella* (Warriner et al., 2002). Moreover, due to the persistence of the pathogenic bacteria, *E. coli* O157:H7 outbreak was likely in a meat grinder that was cleaned and disinfected only once a week (Banatvala et al., 1996). Similarly, *Salmonella* has been persisted in retail butcher shops, kitchen or restaurants that were poorly cleaned and disinfected (Giovannacci et al., 2001). It has been also demonstrated, *E. coli* O157:H7 and *Salmonella* were able to remain in the lairage premises at beef or pork slaughterhouses (Arthur et al, 2008; Small et al., 2006). Plastic, stainless steel, and high density polyethylene that beef contact

surfaces may form biofilms by *E. coli* O157:H7 (Sofos and Geornaras, 2010). The surface of conveyor belt used in pork processing may form easily biofilms by *L. monocytogenes* due to limited disinfectant penetration (Thévenot et al., 2006).

### *Poultry farm*

Many studies have been also conducted to understand the biofilm in poultry industry. *Campylobacter* spp. are a major cause of foodborne disease and are associated with poultry (Trachoo et al., 2001). It has been reported that *Campylobacter jejuni* maintained its viability in biofilm in low-nutrient media and in normal atmospheric conditions for up to 1 week at 10°C, in spite of its susceptibility to oxygen and nutritional stress (Buswell et al., 1998). Therefore, *C. jejuni* can form biofilm in chicken house water systems and infect chickens after biofilm detachment in poultry industry (Trachoo et al., 2001). Besides *Campylobacter* spp., *Salmonella* spp. are also generally found pathogens in poultry industry. The attachment of 69 *Salmonella* strains belonging to 10 serotypes was studied. All *Salmonella* strains formed biofilm on polystyrene. *Salmonella* Agona and *Salmonella* Typhi produced the most substantial biofilm (Díez-García et al., 2012). Kim and Wei (2009) investigated that the main factor to biofilm formation of *Salmonella* Typhimurium. According to study, production of EPS and expression of flagella were associated with the attachment of *S. Typhimurium* on surfaces.

### *Ready-to-eat (RTE) foods*

Ready-to-eat (RTE) foods can be regarded as a relatively high risk food as the products are consumed without any bactericidal processing. As though RTE foods have been well processed, the potential to being contaminated is relatively high (Sofos and Geornaras, 2010). Takahashi et al. (2009) determined that *L. monocytogenes* strains isolated from raw RTE seafood persisted and were able to form a biofilm continually contaminating the food for a prolonged period. Besides, the production of biofilm by *E. coli* O157:H7 on the surface of stainless steel was able to transfer to RTE products (Silagyi et al., 2009). *Listeria* spp. biofilms isolated from RTE food industry that processed franks and lunch meat were also able to transfer to surfaces including conveyors, slicer, hose, and rubber seals (Tompkin, 2002). As a result, the *E. coli* O157:H7 and *L. monocytogenes* could potentially contaminate on food contact surfaces through the formation of biofilm.

#### *Dairy product*

Like other industries, biofilm is believed to be a significant factor of product contamination during dairy processing. For example, the presence of pathogens such as *L. monocytogenes* contaminate the surfaces and induce foodborne diseases in dairy industry (Srey et al., 2013). However, there are different properties in ‘process biofilm’ that is unique to dairy manufacturing plants and forms on surfaces in direct contact with flowing product. Biofilm in other environments take several days or weeks to develop (Stoodley et al., 2002). On the contrary, process biofilm replicates rapidly, with numbers of up to  $10^6$  bacteria per  $\text{cm}^2$

after 12 hours of pasteurization. Besides, a single species often prevails in process biofilm because heat treatment reduces the competition from heat-sensitive bacteria (Bouman et al., 1982). Unlike process biofilm, general biofilm that has same properties in the general food processing environment can also be a problem in dairy industry. When nutrient and temperature conditions are suitable, the cells adhere to surfaces and develop into a biofilm. Therefore, the release of biofilm bacteria into the product may occur during processing (Hinton et al., 2002).

## **1.5. Biofilm control**

### **1.5.1. Conventional methods**

#### *Sodium hypochlorite*

Sodium hypochlorite (NaOCl) is the most widely used disinfectant for bleaching or disinfection in food industry. In many food plants, the bleaching and controlling the microbes are performed with NaOCl at the same time. NaOCl has broad antimicrobial spectrum, induces rapid bactericidal action, and is easy to use. However, there were disadvantages such as irritation to mucous membranes, evolution of toxic chlorine gas when mixed with acid, and decreasing efficacy in the presence of organic load. NaOCl was divided into HOCl or OCl<sup>-</sup> through oxidizing reaction. The bactericidal activity is controlled by the abilities of HOCl and OCl<sup>-</sup> diffusing through the microbial cell membrane (Fukuzaki, 2006).

### *Peracetic acid*

Peracetic acids (PAA) is a product of the reaction between hydrogen peroxide and acetic acid or by the oxidation of acetaldehyde (Srey et al., 2013). PAA has advantages that it does not react with proteins to produce toxic or carcinogenic compounds and decomposes into environmental friendly residues in food, therefore it could be applied without rinsing (Rossoni and Gaylarde, 2000). It was reported that PAA has been effective against biofilm and is favorable to use if the biofilm contains food residues (Chmielewski and Frank, 2003). According to Tote et al. (2010), PAA was active to *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilm cells.

### *Hydrogen peroxide ( $H_2O_2$ )*

$H_2O_2$  has bactericidal and inhibitory activity due to its properties as an oxidant and its capacity to generate other cytotoxic oxidizing species such as hydroxyl radicals. It has been found to be efficient against biofilm and affects the biofilm matrix (Tote et al., 2010).  $H_2O_2$  were used to biofilm removal through degradation of the extracellular network of biopolymers (Christensen et al., 1990). The inactivation effects of 2%, 3%, and 7% hydrogen peroxide on the removal of mature biofilms in dental waterlines were reported by Lin et al. (2011).

### *Ozone*

Ozone is formed by a result that oxygen atoms are exposed to high-voltage electric discharge. This tri-oxygen molecule has strong oxidizing

properties and has been found to be effective to broad spectrum of microorganisms. It could be used as a disinfectant for biofilm as well as planktonic cell (Guzel-Seydim et al., 2004). The microorganisms are eliminated by the disruption of the cell membrane, which leads to the cell lysis. Cell lysis is a faster inactivation mechanism than that of other antimicrobial agents where penetration through the cell membrane is essential to effectively inactivate the microorganisms (Pascual et al., 2007).

### *Ultraviolet radiation*

Ultraviolet (UV) radiation includes a small part of the electromagnetic spectrum that also covers  $\gamma$ -radiation, x-ray, visible light, infrared radiation, microwaves, and radiowaves. UV radiation is divided into three regions: long wave UV-A (320-400 nm), medium wave UV-B (280-320 nm), and short wave UV-C (200-280 nm). Among these, UV-C is considered to be the most effective germicidal to microorganisms such as bacteria, yeast and molds, protozoa, viruses, and algae (Keyser et al., 2007). UV radiation inactivates the microorganisms through formation of pyrimidine dimes in nucleotide bases and DNA damage. These photoproducts twist the sugar phosphate backbone and interrupt proper DNA replication and transcription (Kim et al., 2013). It has been identified as an effective method for disinfection. However, biofilm is hard to treat or frequently relapses when using UV radiation. In order to overcome inefficient conventional UV radiation, pulsed UV-LED device



in which the energy is multiplied many-fold is developed to enhance germicidal effect on biofilm (Li et al., 2010).

### **1.5.2. Novel methods**

#### *Phages*

The phage has been proposed as a biofilm control method. Phage can proliferate at the site of inactivation and then increase the numbers where they are required. Several phages produce enzymes that degrade the EPS of biofilm. A single phage dose should be able to treat a biofilm inactivation as progeny phages infect adjacent cells and degrade the biofilm matrix (Fu et al., 2010). Moreover, phage can be a suitable alternative for chemical-based antimicrobial agents because it does not cause corrosion. In recent study, it was reported that there were >3 log CFU/g reduction in *E. coli* biofilms in egg, meat, cabbage, and lettuce using phages (Jassim et al., 2012). Bacteriophages can generate enzymes, penetrate EPS of *P. aeruginosa* biofilms, and then eventually reduce the biofilm population (Hanlon et al., 2001). Bacteriophage spray treatment was recommended as an alternative for chlorine solution dipping, brushing, or sponging spinach harvester blade according to its comparable inactivation effects (Patel et al., 2011). Recently, a commercial product named Listex P100 was already available to control *L. monocytogenes* (Gracia et al., 2008).

### *Enzymes-based detergents*

Biofilm EPS is composed of various substances such as polysaccharides, proteins, nucleic acids, lipids, and humic substances. The polysaccharides play an important role to maintain cohesiveness of the EPS matrix. Detachment-promoting agents (DW-PAs) of polysaccharide depolymerases, esterases, and dispersin B might break the EPS and then disinfectants could affect the biofilm. Chemicals that change the ionic strength and composition of the liquid medium and affect electrostatic interactions associated with the cohesiveness of the EPS matrix may also be used as DW-PAs including salts or chelating agents (Xavier et al., 2005). Besides, due to the EPS heterogeneity, a mixture of enzymes may be necessary to efficient biofilm degradation. The application on enzymatic cleaning products against biofilms formed by microorganisms such as *Latobacillus bulgaricus*, *Streptococcus thermophilus*, and *Lactobacillus lactis* commonly used in dairy industries (Augustin et al., 2004).

AHLs degradation enzymes called *N* acyl-homoserine lactonases inhibit the signaling molecules of the biofilm-forming bacteria, resulting in QS inhibitory activity against pathogens (Vinoj et al., 2015). The AHLs degradation enzymes have been identified in several bacterial pathogens that produce AHL signals, such as *Agrobacterium tumefaciens* and *P. aeruginosa* (Huang et al., 2003). It was also reported that vanillin showed significant inhibition of AHLs from *Aeromonas hydrophila* biofilms (Ponnusamy et al., 2009).

## *Plasma*

Plasma is a relatively novel approach against a broad range of microbial pathogens either in planktonic cell or biofilm. Plasma is defined as the fourth state of matter. It is generated when supplying energy to gas and includes photons, electrons, positive and negative ions, atoms and free radicals. Among these, it is known that reactive oxygen species (ROS) and reactive nitrogen species (RNS) have a high bactericidal potency. Especially, atomic oxygen, ozone, superoxide, singlet oxygen, peroxide, and hydroxyl radicals included in ROS play an important role in the inactivation mechanism (Ziuzina et al., 2015).

## **2. Plasma**

### **2.1. What is plasma?**

The term plasma is defined as a partially or wholly ionized gas. When the gas is provided sufficient energy, a various active agent, namely UV photons, particles as neutral, positive and negative ions, free electrons, excited atoms and molecules, and free radicals were formed from gas molecules (Tendero et al., 2006). The plasma is more like a gas than any of the other state of matter because the atoms are not in constant contact with each other, but it behaves differently from gas (Fig. 2). Therefore, plasma is considered the fourth state of matter (Nandkumar, 2014). With respect to recent developments in plasma technology, numerous different plasma systems have been fabricated, among these, low temperature

atmospheric pressure plasma has attracted noticeable attraction by the food industry as the non-thermal plasma at atmospheric pressure makes the decontamination process practical and cost-effective (Kim et al., 2013). In addition, low-temperature in plasma discharges makes them suitable for using heat-sensitive products (Gomez et al., 2009).

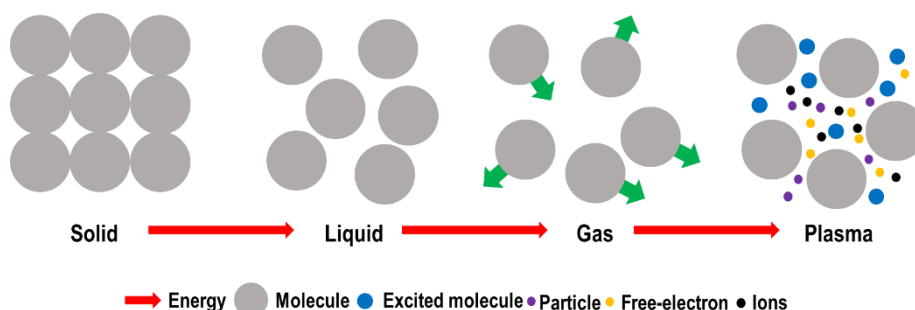


Fig. 2. Schematic representation of plasma

## 2.2. Mechanism of microbial inactivation by plasma

Plasma consists of highly energetic species including UV photons, particles as neutral, positive and negative ions, free electrons, excited atoms and molecules, and free radicals, which are capable of inactivating microorganisms (Tendero et al., 2006). Even though the inactivation effect of plasma is not entirely clarified, there were several studies that propose one of the inactivation mechanisms. UV radiations can cause DNA damage. Strand breaks or structural modifications in DNA such as mutations can cause defective cell proliferate (Muranyi et al., 2010). However, Boudam et al., (2006) reported that UV radiation does not play

a major role in inactivation effect by plasma since the emitted UV radiation from plasma is very low.

The cell wall of bacteria is a primary target for plasma inactivation mechanisms. Reaction between reactive species and components of the cell wall causes critical damage by rupturing the cell wall or changing its chemical or physical properties. Moreau et al. (2008) suggested that OH and NO radicals in humid air cause surface lesions by direct bombardment and local damage by oxidation of cytoplasmic membrane, proteins, and DNA strands. This process called etching. The cell wall bombardment by charged particles (electrons and ions) could break the chemical bonds, erode the membrane, and then plasma toxic compounds penetrate inside the cells. In addition, all of the various plasma active species can synergistically interact. In other words, a combination of the previously referred mechanisms will significantly reinforce the process of inactivation (Gallagher et al., 2007).

### **2.3. Application of plasma treatment**

#### *Animal origin food*

The plasma technology also can act effectively on animal origin food. The application of cold atmospheric pressure plasma for decontamination of an inoculated sliced ready-to-eat meat product (bresaola) was investigated (Rød et al., 2011). Treatments resulted in a reduction of *L. innocua* from  $0.8 \pm 0.4$  to  $1.6 \pm 0.5$  log CFU/g. In another

study, sliced bacon was inoculated with *L. monocytogenes*, *E. coli*, and *S. Typhimurium* (Kim et al., 2011). The samples were treated with atmospheric pressure plasma and two gases, helium or a mixture of helium and oxygen were used for the plasma discharge. Plasma with helium reduced the number of inoculated pathogens by about 1-2 log CFU/g. However, the helium/oxygen gas mixture reduced about 2-3 log CFU/g. The number of total aerobic bacteria showed 1.89 and 4.58 decimal reductions after plasma treatment with helium and the helium/oxygen mixture, respectively (Kim et al., 2011). Similarly, plasma is effective to reduce *C. jejuni* and *S. enterica* on the surface of chicken breast and thigh skin (Dirks et al., 2012). Total log reductions on chicken skin of *C. jejuni* and *S. enterica* were 3.11 and 1.31, respectively, whereas compared to that of chicken breast were 2.45 and 2.45, respectively. Kim et al. (2013) reported that the plasma can be used to enhance the microbiological reduction of pork loin. Pork loin was inoculated initially with *E. coli* and *L. monocytogenes* at 8.54 and 8.10 log CFU/g, respectively. After plasma treatment, *E. coli* count were reduced by 0.34 and 0.55 log cycles and *L. monocytogenes* was also reduced by 0.43 to 0.59 log cycles at 10 min. Jayasena et al. (2015) also revealed that inactivation effects of plasma against *E. coli* O157:H7, *L. monocytogenes*, and *S. Typhimurium* inoculated on pork butt and beef loin samples. The reduction number of bacteria in study ranged from 1.90 to 2.68 log CFU/g after 10 min plasma treatment. A microwave plasma system was used for indirect plasma treatment of fresh porcine musculus *longissimus dorsi* (LD). After plasma treatment, the aerobic

viable count showed that plasma treated *LD* remained constant near the detection limit of 2 log CFU/g whereas the untreated *LD* increased to 9.69 log CFU/g during the storage period of 20 days at 5°C (Fröhling et al., 2012). In addition to, the effect of low temperature plasma system for its capability of killing *E. coli* in milk was examined. A significant 54% reduction in the population of *E. coli* after only 3 min plasma treatment was observed and the initial pre-plasma bacterial count of 7.78 log CFU/mL in whole milk was reduced to 3.63 log CFU/mL after 20 min of plasma application (Gurol et al., 2012).

*S. Enteritidis* and *S. Typhimurium* are two most general causes of foodborne disease and this is mostly contaminated poultry and eggs (Fernández and Thompson, 2012). There were several studies that inactivate the contaminated eggshells by plasma treatment. Ragni et al. (2010) observed that a resistive barrier discharge gas-plasma prototype generating low temperature after-glow gas mixture reduce the *S. Enteritidis* and *S. Typhimurium* on eggshells. A maximum reduction of 2.2-2.5 log CFU/eggshell were showed for *S. Enteritidis* after 90 min of treatment at 35% RH. Moreover, a maximum reduction of 3.8-4.5 log CFU/eggshell were showed after 90 min of treatment at 65% RH and the reinforced effects in increased RH were accounted for the presence of oxygen species as detected in the optimal emission spectra. Furthermore, an important aspect of this study is that plasma treatment did not change the quality of cuticle layer which defenses against microbial penetration. Vannini et al. (2009) also reported that using resistive barrier discharge prototype was developed to inactivate eggs inoculated *S. Enteritidis* and

*L. monocytogenes* and results in the reduction of the pathogens up to 4-5 log CFU/eggshell on eggshell. It revealed that the main species such as OH radicals and NO were responsible for the inactivation activity of the plasma treatment.

### *Biofilm*

Biofilms are problematic in food industry and plasma can be applied to inactivate the contaminated surfaces. Mixed biofilm on stainless steel composed of *S. Typhimurium*, *Staphylococcus epidermidis*, and *Pseudomonas fluorescens* was inactivated by plasma. This study concluded that plasma deposition will be applied on various material used in food processing to reduce bacterial contamination (Denes et al., 2001). The decontamination of a rotating cutting tool used for slicing in the meat industry by means of atmospheric pressure plasmas is investigated. The target is *L. monocytogenes*, a bacterium which causes listeriosis and can be found in plants and food. The non-pathogenic species, *L. innocua*, is used for the experiments. A rotating knife was inoculated with *L. innocua*. The surface of the rotating knife was partly exposed to an atmospheric pressure dielectric barrier discharge (DBD) operated in air, where the knife itself served as a ground electrode. The rotation of the knife ensures a treatment of the whole cutting tool. A 5-log reduction of *L. innocua* is obtained after 340 s of plasma operation. The temperature of the knife after treatment was found to be below 30°C. The design of the device allows a decontamination during slicing operation. Inactivation of *L. innocua* can efficiently be performed by



direct treatment of a material by means of a DBD. The required operation time of the cutting tool was 340 s. This corresponds to an effective treatment time of approximately 41 s, because the DBD does not cover the entire cutting tool. This in turn allows applying this decontamination technique during the slicing process and provides the potential to reduce the risk of cross contamination between separate batches of meat (Leipold et al., 2010).

### *Packaging materials*

Food packaging materials play an important role to preserve food and protect it from outside contamination. However, food materials can be contaminated to bacteria and these contaminants are transferred to food via packages. Conventional methods to inactivate the materials such as chemical solution, UV radiation, or heat have toxic residues, are damaging to materials or often time consuming (Laroussi, 2005). Therefore, plasma treatment of packaging material is an alternative for traditional packaging inactivation. Inoculated by *S. aureus*, *E. coli*, *Clostridium botulinum*, *Bacillus atrophaeus*, and *Aspergillus niger* polyethylene terephthalate (PET) foils were inactivated by DBD plasma. *A. niger* was the most resistant species with an inactivation rate of about 5 log CFU/mL in 5 sec, whereas the other vegetative cells were inactivated at least 6.6 log CFU/mL within 1 sec (Muranyi et al., 2007). Roasting (2013) has patented the method for plasma treatment of plastic bottles. This device allows sterilization during processing. The inactivation of PET foils inoculated by *Bacillus subtilis* spores can be

easily inactivated by low pressure microwave plasmas by 4 orders of magnitudes within a few seconds (Schneider et al., 2005).

#### *Waste water*

The waste water especially using in poultry and meat industries, which demand amount volumes of water was conventionally treated by chlorine-based sanitizers. However, use of chlorine has been concerned about toxicity. Therefore, operator seek new technologies that will assure in safety aspects (Kim et al., 2003). Thus, a pulsed-plasma gas-discharge system was developed for the novel inactivation of chilled poultry wash water. Treatment of poultry wash water in the plasma generation chamber for up to 24 s at 4°C reduced *E. coli*, *C. jejuni*, *Campylobacter coli*, *L. monocytogenes*, *S. enterica* serovar Enteritidis, and *S. enterica* serovar Typhimurium populations to non-detectable levels ( $\leq 8 \log$  CFU/ml). This device generated products such as ozone, UV light, acoustic and shock waves, and pulsed electric fields that have multiple bactericidal properties. Therefore, PPGD offers an exciting complementary or alternative technology for treating raw poultry wash water and for preventing cross-contamination in processing environments (Rowan et al., 2007).

#### **2.4. Plasma treated water (PTW)**

Water treated by plasma called PTW. Especially air plasma discharged by DBD or gliding arc near to non-buffered liquid water at room

temperature and at atmospheric pressure will commonly generate acidic solutions that contain H<sub>2</sub>O<sub>2</sub>, nitrate and nitrite ions, ozone, and other species including reactive oxygen species (ROS) or reactive nitrogen species (RNS) (Traylor et al., 2011). The species generated in liquid are stable for an extended period of time and continue inactivation effect for long period. PTW is more eco-friendly and cost effective than traditional chemical sanitizers that induce carcinogenic compound formation (Xu et al., 2016). The inactivation mechanism was proposed that ROS in PTW damaged the cell membrane, penetrated the membrane of *S. aureus*, then damaged the cell structure, and eventually resulted in the death of *S. aureus* (Zhang et al., 2013). Besides, it has been reported that this acidic solution is effective to microbial inactivation. The inactivation efficacy associated with H<sub>2</sub>O<sub>2</sub> and nitrite was measured in both 15 min and 3 h exposures with PTW. Log reduction number in *E. coli* K12 was greater in 3 h exposures PTW than that of 15 min exposure PTW. It was suggested that the inactivation activity of acidified nitrite is due to the decomposition of nitrous acid to generate nitric oxide (Traylor et al., 2011). The synergistic effect of acidified nitrite with H<sub>2</sub>O<sub>2</sub> and nitrate enhanced the inactivation activity of PTW. The formation of reactive species that results from the reaction between nitrite and H<sub>2</sub>O<sub>2</sub> such as peroxynitrite and nitrogen-oxide products influenced the inactivation activity (Naïtali et al., 2012). Oehmigen et al. (2011) observed the pH decreased in PTW and it was concluded that nitric acid formation from plasma-generated reactive nitrogen species was the main source of liquid acidification. Inactivation effects of plasma were significantly

accelerated if pH decreased after plasma treatment, whereas acidic conditions alone did not explain whole inactivation activity of bacteria. This result was supported that increasing H<sub>2</sub>O<sub>2</sub> concentration increased the inactivation activity of PTW. Consequently, synergistic action of both reactive oxygen and nitrogen species was responsible for inactivation effects of plasma. After the basic studies, PTW was applied to food industry. PTW treatments reduced the number of *S. aureus* inoculated on strawberries. The inactivation activity was responsible for oxidative stress induced by ROS in PTW. In addition, there was no significant change in color, firmness, and pH of PTW treated strawberries. Therefore, PTW can be an alternative disinfectant to conventional sanitizers applied in the food industry (Ma et al., 2015). PTW reduced the microbial counts in button mushrooms, *Agaricus bisporus* by 1.5 and 0.5 log CFU/g for bacteria and fungi during storage, respectively. Besides, there was no significant change in firmness, color, pH and antioxidant properties in button mushrooms. Thus, PTW is a promising method for postharvest fresh-keeping of button mushrooms (Xu et al., 2016).

### **III. Effect of Different Atmospheric Pressure Plasma Treatment Methods on Pathogenic Bacteria in Biofilm and Its Inactivation Mechanism**

#### **ABSTRACT**

The objective of this study was to investigate the optimum conditions of inactivation efficiency of various atmospheric pressure plasma (APP) treatment methods to biofilms and furthermore, the inactivation mechanism of optimized APP treatment was investigated. *Listeria monocytogenes* (Gram-positive bacteria) and *Escherichia coli* O157:H7 (Gram-negative bacteria) biofilms were formed on stainless steel, widely used material for various utensils in food industry. Then, the biofilms were treated by four different methods of plasma treatment for 10 min: 1) direct plasma treatment (Direct-P); 2) plasma treatment dissolved in distilled water (DW-P), 3) 100 ppm of sodium chloride (NaCl-P), and 4) 100 ppm of sodium hypochlorite (NaOCl-P). No significant difference was shown in the reduction number ( $\log \text{CFU}/\text{cm}^2$ ) of *L. monocytogenes* biofilms by different plasma treatment methods. However, the reduction number of *E. coli* O157:H7 was effective in order as NaOCl-P (3.45 log) > Direct-P (2.26 log) = NaCl-P (2.07 log) = DW-P (1.96 log). In NaOCl-P environment, the concentrations of hydroxyl radicals and peroxyxynitrite were the highest among the different methods of plasma treatment,

indicating that these chemical species play a major role in inactivation of pathogenic bacteria in biofilms. Therefore, NaOCl-P is the optimum plasma treatment methods for inactivation of *E. coli* O157:H7 biofilms formed on stainless steel. In addition, the merit of using lower concentration of NaOCl than typical practice in industry can be achieved.

*Keywords:* *Escherichia coli* O157:H7, *Listeria monocytogenes*, Biofilm, Atmospheric pressure plasma, Inactivation mechanism, Chemical species

# 1. Introduction

Consumers have been concerned about the microbial safety of food. Therefore, there are many efforts for prevention and control of contamination of microorganism in food industry. Despite the efforts, the Centers for Disease Control and Prevention reported that the foodborne disease causes 325,000 hospitalizations and generating 5,000 deaths in the U.S. annually (Schlisselberg and Yaron, 2013). Generally, foodborne diseases occur when using contaminated food materials by foodborne pathogens or practicing improper disinfection treatment during production due to cross-contamination.

Inappropriate disinfection of surfaces in food plant contributes the development of bacterial biofilms (Ban and Kang, 2016). The cross-contamination through biofilms is responsible for over 65% of all microbial diseases (Potera, 1999). A biofilm is a sessile bacterial community of microbial cells formed on biotic and abiotic surfaces such as plastic, rubber, glass, cement and stainless steel (Schlisselberg and Yaron, 2013). The formation of biofilm is initiated by bacterial adhesion to a surface. Bacteria attachment stimulates extracellular polymeric substances synthesis by bacteria that plays a major role to increase resistance surviving in the extreme environments (Steenackers et al., 2012). Stainless steel, used as an important material in kitchen or plant, is a major cause for biofilm formation. (Bae et al., 2009). In meat and dairy industries, the stainless steel equipment such as meat grinder, pipelines, and other utensils could be a potential harbor for foodborne

diseases due to pathogenic bacteria (Banatvala et al., 1996; Michaels et al., 2001). Thus, it is necessary to inactivate the bacteria in biofilms in the food industry to prevent the outbreak of food-borne disease caused by the cross-contamination.

The conventional sanitizer used to disinfect biofilms in food processing was sodium hypochlorite (NaOCl). The U.S. FDA allowed maximum concentration of 200 ppm (Gulati et al., 2001) and thus, 200 ppm of NaOCl solution is widely used in food industries (Kondo et al., 2006). NaOCl has advantages to be cheap and effective in a broad microbial spectrum. However, the formation of by-products such as trihalomethanes and haloacetic acids has been considered harmful to public health (Haute et al., 2015). As for the increase in concerns about disadvantages of NaOCl, the alternative technologies have been being searched (Fernandez et al., 2013).

Plasma is innovative non-thermal sterilization technology for inactivation of microorganisms. Plasma consists of electrons, photons, positive and negative ions, neutral atoms, free radicals, UV photons and reactive species. These chemical species have been reported to play a major role in inactivation of microorganism (Yong et al., 2015). The inactivation efficiency of atmospheric pressure plasma (APP) is different from experimental conditions (Yong et al., 2014), thus, there were studies that using various methods of plasma treatment to find the optimum inactivation efficiency such as direct plasma treatment, post-duration after plasma treatment, and plasma treated water (PTW).



Direct plasma treatment is that discharged plasma is directly contact with sample surfaces to effectuate the potent inactivation effect (Bauer et al., 2017). There were studies that have been investigated on inactivation of microorganisms by direct plasma such as *Pantoea agglomerans* on bell peppers (Vleugels et al., 2005). Post-duration effect is that plasma is discharged in sealed conditions and the samples were kept for post-duration time to contact with retained chemical species even after plasma discharge was switched off (Han et al., 2016). Yong et al. (2015) and Laurita et al. (2015) reported that there was bacterial inactivation effect by post-treatment duration referring to trap the chemical species after switched off after certain period of treatment. Recently, the studies related to water treated by plasma called PTW has been conducted. The PTW creates an acidified solution containing chemical species which play a role in the inactivation of microorganisms (Shen et al., 2016). Thus, it has been reported that the PTW can also be an efficient disinfectant (Zhang et al., 2013).

However, the study related to different plasma treatment methods for inactivation of pathogenic biofilms on stainless steel was limited. To apply the plasma technology in industry, the optimization process is necessary. Therefore, the objective of this study was to investigate the optimum conditions for inactivation of bacterial biofilms on stainless steel using APP treatment methods including direct plasma treatment and plasma treated solution (PTS) included distilled water (DW), 100 ppm NaCl, and 100 ppm NaOCl. Furthermore, the major chemical species in inactivation of bacterial biofilms were identified.

## 2. Materials and methods

### 2.1. Bacterial strains and culture preparation

*E. coli* O157:H7 (KCCM 40406, Gram-negative) and *L. monocytogenes* (KCTC 3569, Gram-positive) were cultivated independently in tryptic soy broth (TSB; Difco, Detroit, USA) and TSB containing 0.6% yeast extract (Difco), at 37°C for 48 h, respectively. Each strain was transferred to a 50 mL conical centrifuged tubes and centrifuged at  $2,149 \times g$  for 20 min at 4°C in a refrigerated centrifuge (HM-150IV, Hanil Co. Ltd., Incheon, Korea), respectively. The pellet was washed twice with sterile saline (0.85%). The final pellets of each strain were resuspended in sterile saline, corresponding to approximately  $10^7$ - $10^8$  log CFU/mL.

### 2.2. Preparation of stainless steel coupons and biofilm formation

Stainless steel coupons (20 × 20 × 10 mm) were used in this study because it is widely used material for various utensils in meat and food industry. The coupons were soaked in sterile DW for 10 min to remove debris and grease on surface. The rinsed stainless steel coupons were sterilized using autoclave before use. The rinsed stainless steel coupons were transferred to clean flasks containing fresh broth in 50 mL flask and 100 µL bacteria suspension prepared as described above. The stainless steel coupons with the bacteria were incubated at 37°C with gently

shaking to facilitate attachment of cells. The coupons were removed from the broth after 7 days (culture medium was replaced every other day). After incubation, coupons were rinsed two times with sterile saline to remove non-attached cells and thoroughly dried for 1 h in clean bench before use.

### *2.3. Treatment with atmospheric pressure plasma*

A plasma source is designed for generating APP within the plastic container ( $137 \times 104 \times 53$  mm) (Yong et al., 2015). The powered electrode and ground electrode were made of copper and polytetrafluoroethylene sheet, respectively. To examine the inactivation effect of pathogens, the stainless steel coupons contained petri dish ( $90 \times 15$  mm) were placed at the bottom of the container. A sine-waveform voltage of 4.2 Vpp and frequency of 2.5 kHz were applied to powered electrode, whereas the other electrode was grounded. The atmospheric air was used as an operating gas. The control coupons were prepared as the same condition without plasma treatment. The coupons were treated by four types of different plasma treatment methods in this study: 1) direct plasma treatment (Direct-P); plasma treatment with 2) DW (DW-P) only; 3) 100 ppm NaCl solution (NaCl-P); and 4) 100 ppm NaOCl solution (NaOCl-P) for 10 min, respectively. NaCl and NaOCl was considered as chloride ion ( $\text{Cl}^-$ ) donor which may have pathogen inactivation activity (Estrela et al., 2002) Moreover, to investigate the influence of post-treatment storage duration, the samples were placed in

the container with the lid closed for 10 min (Closed state) after plasma discharge was switched off. These post-duration treatments were applied at all samples and compared to samples with lid opened for 10 min immediately after plasma discharge was switched off (Opened state).

#### *2.4. Microbiological analysis*

After plasma treatment, each stainless steel coupons were transferred to sterile 50 mL conical centrifuge tubes containing 9 mL sterile saline and blended for 30 min using shaking bath. Immediately after shaking, cell suspensions were 10-fold serially diluted in sterile saline. The media used for *E. coli* O157:H7 and *L. monocytogenes* were tryptic soy agar (TSA; Difco, Detroit, USA) and TSA containing 0.6% yeast extract (Difco), respectively. The 100  $\mu$ L of cell suspensions were spread on appropriate medium and the plates were incubated at 37°C for 48 h. All colonies were counted and the number of microorganisms was represented to log CFU/cm<sup>2</sup> and the reduction bacterial number was observed.

#### *2.5. Confocal laser scanning microscopy (CLSM)*

A BacLight Live/Dead Bacterial Viability Kit (L-7012, Molecular Probes, Eugene, USA) was used to examine cell membrane integrity. The kit consists of two nucleic acid stains, SYTO 9 and PI to stain viable

cells and damaged or dead cells, respectively. Viable cells appeared green in color, whereas damaged or dead cells were stained red. The two stains, SYTO 9 and PI, dissolved in dimethyl sulfoxide, were mixed together (3  $\mu$ L + 3  $\mu$ L) and diluted in DW (1 mL). For analyzing CLSM, biofilm stainless steel coupons treated by plasma were stained with 2 mL of each staining mixture and incubated in this solution in darkness at  $20 \pm 1^\circ\text{C}$  for 15 min. After incubation, each coupon was mounted on cover slips for analysis with Leica TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany). Randomly selected areas of each sample were imaged using  $\times 100$  magnification. Green and red fluorescence were captured with a CLSM using excitation/emission wavelengths of 488/530 and 568/630 nm, respectively.

## 2.6. Scanning electron microscope (SEM)

For the SEM analysis, additional *E. coli* O157:H7 and *L. monocytogenes* biofilm on stainless steel coupons from each sample and treatment (untreated, Direct-P, and NaOCl-P) were obtained. A vapor fixation procedure was employed in present study to preserve and examine biofilm on stainless steel. The biofilms on stainless steel were fixed with Karnovsky's fixative and kept closed at room temperature for 2 h. The specimens were also exposed in a sealed petri dish to the vapor of 2% osmium tetroxide for at least 2 h. Afterwards, the specimens were mounted on a metal stub and coated with white gold in an EM ACE200 equipment (10 nm, 30 mA, 2 min). Examinations were carried out with

a scanning electron microscope SUPRA at 2 kV and at working distance range from 3.5 to 4.0 mm (Carl Zeiss, Oberkochen, Germany).

### *2.7. Visible emission spectrum in direct plasma treatment*

The visible emission spectrum of the plasma discharge for direct plasma treatment was obtained by a spectrometer (MAYA2000 Pro, Ocean Optics, Inc., FL, USA) equipped with the relevant optical setups (Yong et al., 2015).

### *2.8. pH and oxidation reduction potential (ORP) of PTS*

The pH and ORP of PTS were immediately measured after plasma treatment. pH was measured by a pH meter (Seven Go, Mettler-Toledo, Greifensee, Switzerland) and ORP was measured by an ORP meter (Orion 4 star, Thermo Fisher Scientific Inc., Beverly, USA).

### *2.9. Analysis of chemical species in PTS*

After plasma treatment, 1.5 mL of PTS was filtered by syringe filter (0.2  $\mu$ M). Nitrite ( $\text{NO}_2^-$ ), nitrate ( $\text{NO}_3^-$ ), and chloride ion ( $\text{Cl}^-$ ) were determined by a Dionex ICS-3000 ion chromatography (Dionex Corporation, Sunnyvale, USA). The ion chromatography was equipped with an dual eluent generator system, dual chromatography compartment

with dual suppressed conductivity detectors, dual gradient pump, autosampler. Samples were separated with an IonPac AS20 analytical column (250 mm  $\times$  4 mm I.D., Dionex Corporation, Sunnyvale, USA) and its respective guard column (AG 20, 50 mm  $\times$  2 mm I.D., Dionex Corporation, Sunnyvale, USA) with a flow rate of 1 mL/min and a thermostatted temperature of 30°C. A ASRS URTRA II (4 mm) self-regenerating suppressor was used for suppressed conductivity detection. A 25  $\mu$ L sample injection volume was used throughout the experiment. The gradient elution conditions consisted of 15 mM KOH from 0 to 8 min, 40 mM from 8 to 18 min, then, 15 mM from 19 to 20 min.

Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) concentration was determined by a spectrophotometric method using ammonium metavanadate (99%, Sigma-Aldrich GmbH, Sternheim, Germany), which immediately changes the color to a red-orange due to peroxovanadium cation formed during the reaction of hydrogen peroxide with metavanadate. Ammonium metavanadate (10 mM) was prepared under magnetic stirring and at 50°C until complete dissolution. After complete dissolving, 1 mL of ammonium metavanadate and 0.3 mL of 5 M sulfuric acid (95%, Junsei Chemical Co. Ltd., Chuo-ku, Japan) were added slowly to 1 mL of PTS. After 2 min, the absorbance of the vanadate solution was measured at 450 nm using a spectrophotometer (X-ma 3100, Human Co. Ltd., Seoul, Korea).

Hydroxyl radicals ( $\text{OH}\cdot$ ) in PTS were measured by the formation of phenol from benzene. Through the reaction with hydroxyl radicals, the

benzene can be directly converted into phenol. Therefore, hydroxyl radicals concentration was estimated from phenol which is an oxidized product of benzene. Benzene (8 mM, 8 mL) was added to 8 mL of each DW, 100 ppm NaOCl, or 100 ppm NaOCl solution, respectively and total 16 mL of solutions were treated with APP (Laurita et al., 2015).

Peroxynitrite ( $\text{ONOO}^-$ ) content was measured by the formation of 4-hydroxy-3-nitrophenylacetic acid ( $\text{NO}_2\text{HPA}$ ) from 4-hydroxyphenylacetic acid (HPA). Peroxynitrite is converted to nitronium ion, which can form nitrated phenolic compounds such as HPA. Thus, peroxynitrite concentration was evaluated from  $\text{NO}_2\text{HPA}$  which is a nitrated product of HPA. HPA (12 mM, 8 mL) was added to 8 mL of DW, 100 ppm NaOCl, or 100 ppm NaOCl solution and total 16 mL of solutions were treated with APP (Fukuyama et al., 1996).

After plasma treatment, phenol and  $\text{NO}_2\text{HPA}$  concentration were determined by Thermo Ultimate 3000 HPLC (Dionex Corporation, Sunnyvale, USA). The analytical column was a VDSpher PUR C18 capillary column ( $4.6 \times 250$  mm; VDS optilab, Berlin, Germany) and maintained at a temperature of  $30^\circ\text{C}$  by means of a column oven. Mobile phase B consisted of 100% acetonitrile and mobile phase C consisted of water containing 0.1% trifluoroacetic acid.

## *2.10. Microbiological inactivation ability of chemical species*

*E. coli* O157:H7 and *L. monocytogenes* of 100  $\mu\text{L}$  bacteria suspension



prepared as described above were transferred to microtube containing 1 mL of nitrite (3 and 5 mg/L, Sigma-Aldrich GmbH), nitrate (80 and 100 mg/L, Sigma-Aldrich GmbH), and hydrogen peroxide (100 and 150 mg/L, Junsei Chemical Co. Ltd.) solution. The media used for *E. coli* O157:H7 and *L. monocytogenes* were tryptic soy agar (TSA; Difco, Detroit, USA) and TSA containing 0.6% yeast extract (Difco), respectively. The 100 µL of each dilution on plates were spread on appropriate medium. The plates were incubated at 37°C for 48 h. All colonies were counted and the reduction number of microorganisms was represented to log CFU/cm<sup>2</sup>.

### *2.11. Statistical analysis*

All experiments were repeated three times except for CLSM and SEM, with independently prepared samples. Statistical analysis was performed by one-way analysis of variance using the procedure of General Linear Model and the Statistical Analysis System (SAS, Release 9.3, SAS Institute Inc., Cary, USA). The significant differences between mean values were tested by Student-Newman-Keul's multiple comparison test with a probability level of  $P < 0.05$ . Mean values and standard errors of the means (SEM) were reported.

### 3. Results and discussion

#### 3.1. Microbiological analysis

Table 1 shows the inactivation of *E. coli* O157:H7 and *L. monocytogenes* biofilms by different APP treatment methods. The reduction number (log CFU/cm<sup>2</sup>) of *E. coli* O157:H7 was determined in order as NaOCl-P (3.04 log) > NaCl-P (2.03 log) = Direct-P (1.99 log) = DW-P (1.91 log) in opened state and NaOCl-P (3.45 log) > Direct-P (2.26 log) = NaCl-P (2.07 log) = DW-P (1.96 log) in closed state. However, there were no differences in reduction number by different APP treatment methods in *L. monocytogenes* biofilms, except for DW-P. When comparing the reduction number between *E. coli* O157:H7 and *L. monocytogenes*, the reduction number of *E. coli* O157:H7 biofilms was higher than that of *L. monocytogenes*. Previous study showed that Gram-positive bacteria have a thick peptidoglycan structure on the outside of the cell and it makes more resistant to morphological changes. On the other hand, Gram-negative bacteria is more vulnerable than Gram-positive due to a unique outer membrane in their cell envelope and thin peptidoglycan structure (Laroussi, 2002). The penetration across a thick cell wall of Gram-positive would be slower than across a thin Gram-negative bacteria, leading to a difference in APP resistance (Mai-Prochnow et al., 2016). It was demonstrated that the hydroxyl radicals and atomic oxygen produced by APP lead to breakage of C-O, C-N, and C-C bond in peptidoglycan structure (Mai-Prochnow et al., 2016). Han et al. (2016) reported that the cell envelope is the major target of reactive

species in Gram-negative bacteria. The authors reported that through the APP treatment, cell envelope is disrupted and results in cell leakage in Gram-negative bacteria. On the other hand, in Gram-positive bacteria, reactive species can penetrate cell membranes by pores in the membrane or active transport across the lipid bilayer and cause damage to intracellular components (e.g., DNA). It was also reported that there was rapid rupture of the membrane of Gram-negative bacteria following exposure to plasma, whereas Gram-positive bacteria did not show visible morphological changes (Laroussi, 2002). Unlike present study, however, there was no difference between the number of *E. coli* (Gram-negative) and *S. aureus* (Gram-positive) after treatment of APP (Han et al., 2016). Thus, it is still controversial to inactivation efficiency of APP against Gram-negative and Gram-positive bacteria.

**Table 1.** Log reduction number (log CFU/cm<sup>2</sup>) of *E. coli* O157:H7 and *L. monocytogenes* inoculated on stainless steel treated with atmospheric pressure plasma

		Treatment				SEM <sup>1</sup>
		Direct-P*	DW-P*	NaCl-P*	NaOCl-P*	
<i>E. coli</i> O157:H7	Opened*	1.99 <sup>b</sup>	1.91 <sup>b</sup>	2.03 <sup>b</sup>	3.04 <sup>ay</sup>	0.085
	Closed*	2.26 <sup>b</sup>	1.96 <sup>b</sup>	2.07 <sup>b</sup>	3.45 <sup>ax</sup>	0.103
	SEM <sup>2</sup>	0.107	0.055	0.124	0.077	
<i>L.</i> <i>monocytogenes</i>	Opened	0.52	0.38 <sup>y</sup>	0.44	0.54	0.059
	Closed	0.60	0.52 <sup>x</sup>	0.46	0.55	0.038
	SEM <sup>2</sup>	0.041	0.031	0.082	0.020	

\*Direct-P, direct plasma treatment; DW-P, distilled water treated with plasma; NaCl-P, 100 ppm NaCl treated with plasma; NaOCl-P, 100 ppm NaOCl treated with plasma; Opened, the lid was opened immediately after plasma treatment; Closed, the lid was closed for 10 min after plasma treatment.

<sup>1</sup>Standard error of the means (n=12), <sup>2</sup>(n=6).

<sup>a,b</sup>Different letters within the row differ significantly ( $P < 0.05$ ).

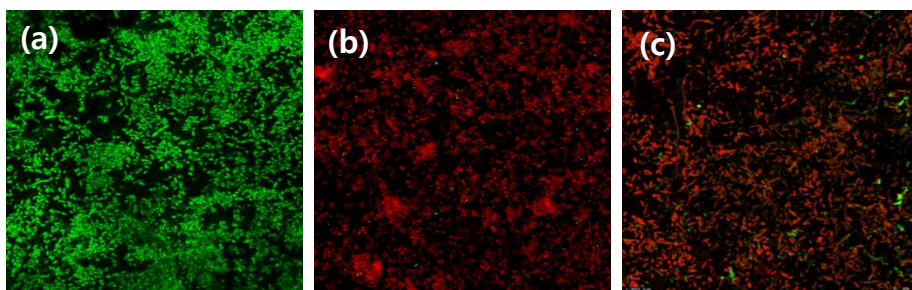
<sup>x,y</sup>Different letters within the column differ significantly ( $P < 0.05$ ).

### 3.2. Confocal laser scanning microscope (CLSM) and scanning electron microscope (SEM)

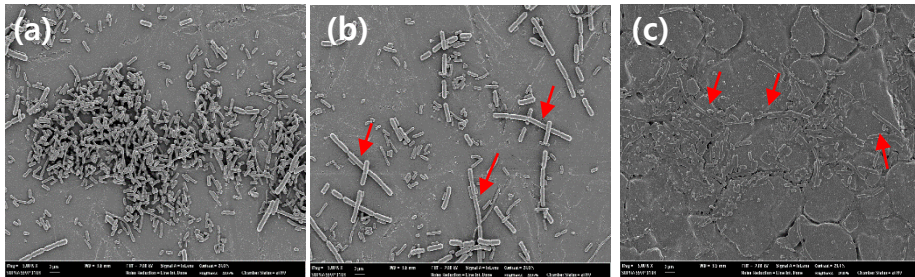
The cell membrane of *E. coli* O157:H7 biofilms was observed by CLSM (Fig. 3) to see the inactivation by visual conformation. Especially, *E. coli* O157:H7 biofilms treated with NaOCl-P in closed state was designated due to the highest in the reduction number of bacteria in biofilms among PTS. Untreated *E. coli* O157:H7 biofilms were stained mostly green as shown in Fig. 3(a), whereas the range of cell stained by red color increased in Direct-P and NaOCl-P treatment [Fig. 3(b) and (c)]. The staining result suggested that most biofilm cells were remained damaged by Direct-P and NaOCl-P treatment. However, no significant difference was shown in CLSM of biofilms between Direct-P and NaOCl-P treatment. The untreated *E. coli* O157:H7 biofilms cells were dispersed in single bacterial cells morphorogically [Fig. 3(a)]. On the other hand, it was observed a number of long-stick structures of *E. coli* O157:H7 and in Direct-P and NaOCl-P treatment. [Fig. 3(b) and (c)].

Similarly, compared to the untreated coupons which shows dispersed to single bacterial cells, the plasma treated *S. Typhimurium* formed aggregated long structures which might result from incomplete cell division (Park et al., 2016). The elongation of *Pseudomonas aeruginosa* is due to incomplete septum formation, which is influenced by nitric oxide (NO $\cdot$ ). However, the significant mechanism of how the nitric oxide can stimulate the cell elongation was not explained clearly so far (Yoon et al., 2011).

SEM micrographs were observed for a more detailed image for the pathogenic biofilms. In untreated coupons, most of bacteria formed biofilms were single bacteria [Fig. 4(a)]. With APP treated samples, it was identified *E. coli* O157:H7 on stainless steel coupons tends to forming long-stick structures [Fig. 4(b) and (c)].



**Fig. 3.** Membrane integrity of *E. coli* O157:H7 biofilms on stainless steel observed by CLSM. The *E. coli* O157:H7 biofilms were treated with the lid was closed for 10 min after plasma treatment and (a) Untreated, (b) direct plasma treatment (Direct-P), (c) 100 ppm NaOCl solution treated with plasma (NaOCl-P).

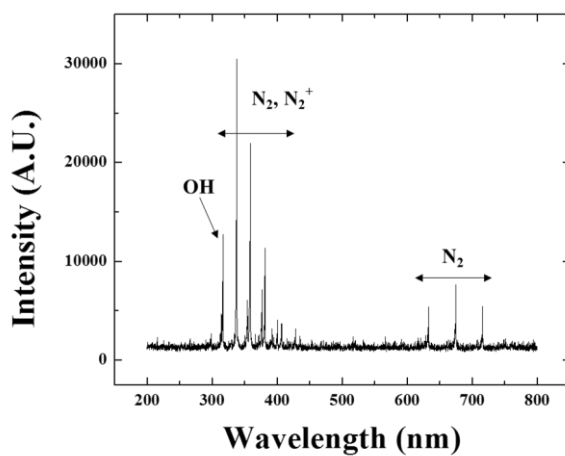


**Fig. 4.** Scanning electron microscope micrographs of *E. coli* O157:H7 biofilms on stainless steel. The *E. coli* O157:H7 biofilms were treated with the lid was closed for 10 min after plasma treatment and (a) Untreated, (b) direct plasma treatment (Direct-P), (c) 100 ppm NaOCl solution treated with plasma (NaOCl-P). Red arrows indicate the long-stick structures of bacteria.



### *3.3. Visible emission spectrum*

Visible emission spectrum was used to determine the main excited active species generated by APP ranging from 200 to 800 nm in Direct-P environment. The emission spectrum is presented in Fig. 5. Due to the ambient air, the following related oxygen and nitrogen lines were presented in the emission spectrum. In general, the dominant radicals present in APP discharge are nitric oxide ( $\text{NO}\cdot$ ) and hydroxyl radicals ( $\text{OH}\cdot$ ) when humid air serves as the process gas (Kamgang-Youbi et al., 2009). These radicals are precursors of other chemical species in solution, e.g., nitrite, nitrate, and hydrogen peroxide (Kamgang-Youbi et al., 2009).



**Fig. 5.** Emission spectrum of the atmospheric pressure plasma in direct plasma treatment (Direct-P) environment. OH, N<sub>2</sub>, and N<sub>2</sub><sup>+</sup> molecular spectra generated because of the ambient air are observed.

### 3.4. pH and oxidation reduction potential (ORP) of PTS

The pH value and ORP of the PTS are shown in Table 2. The pH value of untreated DW was 6.59 (data not shown) and that of PTS was decreased compared to untreated DW. The pH values in opened state was determined in order as NaOCl-P (5.37) > DW-P (3.54) = NaCl-P (3.24), whereas there was no difference in closed state. Generally, plasma treatment led to the acidification of solution due to chemical species. For example, there is a reduction of pH when existing of hydroxyl radicals (Estrela et al., 2002) and acidification of PTW also can be explained existence of nitrite and nitrate (Machala et al. 2013). Oehmigen et al. (2010) reported that the acidification of solution could be explained as a result of the formation of nitrous acid ( $\text{HNO}_2$ ) and nitric acid ( $\text{HNO}_3$ ). The generation of acidic  $\text{H}_3\text{O}^+$  ions by reactions of water molecules with hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) generated by plasma could also influence the acidification of PTS. Ikawa et al. (2010) found that inactivation of bacteria such as *S. aureus* and *E. coli* was dependent on acidification of solution treated. Unlike previous study, however, acidification alone cannot explain the inactivation of biofilms on stainless steel observed in present study because NaOCl-P was higher pH but obtained the greatest reduction number.

At first, ORP was evaluated to measure the general level of chemical species in PTS. ORP of untreated DW was 597.8 mV and that of PTS was increased to 1087.90 and 1076.90 mV when NaOCl-P opened and closed state, respectively. No significant difference was shown between

opened and closed state. Kim et al. (2000) suggested that the ORP of solution might be the main factor influencing microbial inactivation. The ORP of NaOCl-P in this study was greater than 1,000 mV and it can influence the inactivation of bacteria in biofilms because a high ORP can influence the bacterial defense mechanism and the outer and inner membranes of the bacteria (McPherson, 1993). The range of ORP favored by aerobic bacteria to grow is +200 to +800 mV (Venkitanarayanan et al., 1999). Therefore, the increase of ORP indicates that a large amount of chemical species could be generated in NaOCl-P compared to DW-P and NaCl-P, which results in increase of the inactivation efficiency of bacteria in biofilms in the present study.

**Table 2.** pH and oxidation reduction potential (ORP) of atmospheric pressure plasma treated solution

Treatment	Post-duration		SEM <sup>1</sup>
	Opened*	Closed*	
pH			
DW-P*	3.54 <sup>ay</sup>	2.94 <sup>b</sup>	0.047
NaCl-P*	3.24 <sup>y</sup>	3.21	0.142
NaOCl-P*	5.37 <sup>x</sup>	4.60	0.760
SEM <sup>2</sup>	0.481	0.410	
ORP (mV)			
DW-P	653.63 <sup>y</sup>	659.70 <sup>y</sup>	13.139
NaCl-P	663.83 <sup>y</sup>	674.70 <sup>y</sup>	8.321
NaOCl-P	1087.90 <sup>x</sup>	1076.90 <sup>x</sup>	18.016
SEM <sup>2</sup>	7.132	18.076	

<sup>\*</sup>DW-P, distilled water treated with plasma; NaCl-P, 100 ppm NaCl treated with plasma; NaOCl-P, 100 ppm NaOCl treated with plasma; Opened, the lid was opened immediately after plasma treatment; Closed, the lid was closed for 10 min after plasma treatment.

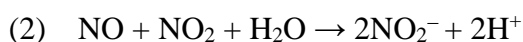
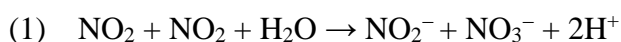
<sup>1</sup>Standard error of the means (n=6), <sup>2</sup>(n=9).

<sup>a,b</sup>Different letters within the row differ significantly ( $P < 0.05$ ).

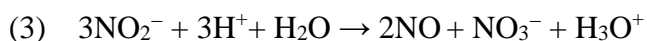
<sup>x,y</sup>Different letters within the column differ significantly ( $P < 0.05$ ).

### 3.5. Analysis of chemical species in PTS

Nitrite ( $\text{NO}_2^-$ ) concentration was higher in opened state compared to closed state in DW-P, NaCl-P, and NaOCl-P. In opened state, the concentration of nitrite in DW-P was 2.02 mg/L which was the highest concentration compared to other treatments. In closed state, the concentration of nitrite in NaCl-P was 0.56 mg/L which is higher than that of DW-P and NaOCl-P. Nitrate ( $\text{NO}_3^-$ ) concentration was also higher in opened state compared to closed state in all treatments. The concentration of nitrate in NaOCl-P was 96.09 and 38.10 mg/L in opened and closed state, respectively (Fig. 7). Machala et al. (2013) reported that the formation of nitrite and nitrate in PTW can change the pH towards acidic range and enhance plasma inactivation efficiency. The formation of nitrite and nitrate in the PTS, through dissolution of  $\text{NO}_x$  in solution leads to the decrease of pH (Equation 1 and 2).

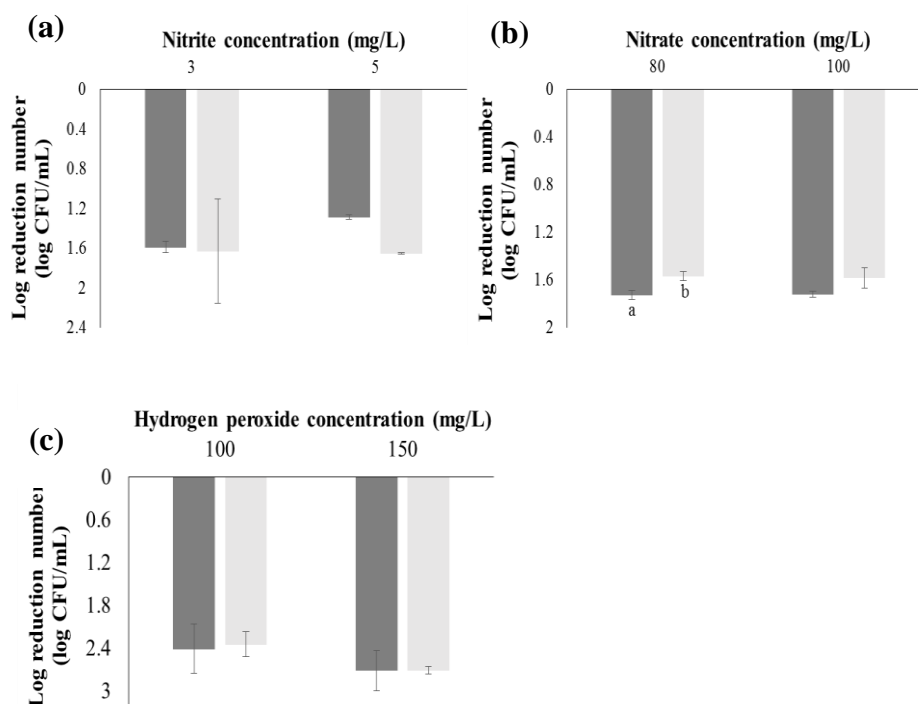


Similarly, to present study, Machala et al. (2013) also reported that the concentration of nitrite was relatively lower compared to nitrate and it can be explained by a transformation of nitrite to nitrate at acidic conditions (Equation 3).



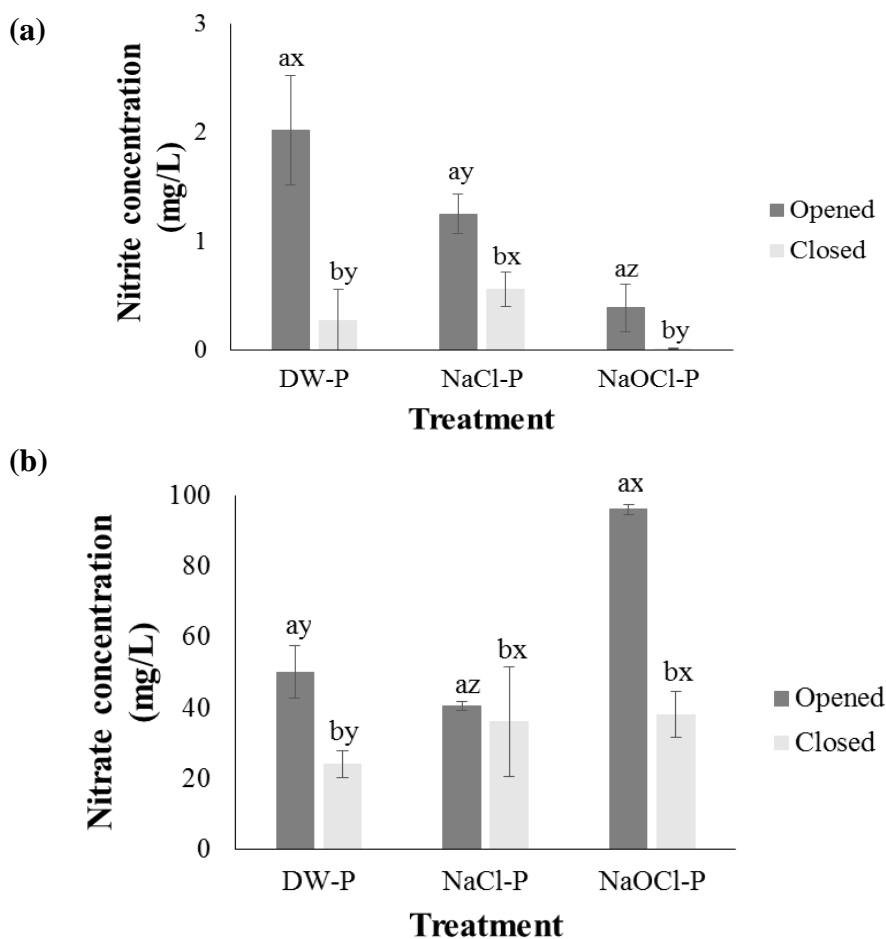
Based on these previous results, the present study conducted the

microbiological analysis to investigate the inactivation of bacteria by nitrite and nitrate. Nitrite and nitrate can reduce the number of bacteria in the range of 1.4-1.7 log reduction at designated concentrations. The concentrations of the individual chemical species were prepared by the range generated in present plasma devices (Fig. 6). Such supporting effect of bacterial inactivation by nitrite and nitrate can explain the increasing reduction number of bacteria on biofilms. However, the concentration of nitrite and nitrate alone cannot explain the present result as the reduction number of bacteria was higher in NaOCl-P in closed state compared to other treatments, whereas the concentration of nitrite and nitrate in PTS was not consistently higher in NaOCl-P environment (Table 1).



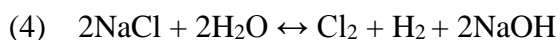
**Fig. 6.** Log reduction number (log CFU/mL) of *E. coli* O157:H7 and *L. monocytogenes* after treated by different chemical species solution. (a) nitrite, (b) nitrate, (c) hydrogen peroxide. <sup>a,b</sup>Different letters among the same concentration indicate significant difference among different bacteria





**Fig. 7.** Concentration (mg/L) of reactive species in plasma treated solution by different atmospheric pressure plasma methods. Abbreviation: DW-P, distilled water treated with plasma; NaCl-P, 100 ppm NaCl treated with plasma; NaOCl-P, 100 ppm NaOCl treated with plasma; Opened, the lid was opened immediately after plasma treatment; Closed, the lid was closed for 10 min after plasma treatment. (a) nitrite, (b) nitrate <sup>a,b</sup>Different letters among the same solution differ significantly. <sup>x-z</sup>Different letter among the same post-duration treatments differ significantly ( $P < 0.05$ ).

Fig. 8 shows the concentration of chloride ion ( $\text{Cl}^-$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in PTS. The concentration of chloride ion in NaCl-P was 68.75 and 79.86 mg/L in opened and closed state, respectively. NaCl and NaOCl solution can be electrolyzed by plasma treatment (Sakiyama et al. 2009). The electrolyzed solution, which includes high free chlorine species such as hypochlorous acid ( $\text{HOCl}$ ), hypochlorite ion ( $\text{OCl}^-$ ), dissolved  $\text{Cl}_2$ , and chloride ion and reactive species such as hydroxyl radicals, is considered to be effective for inactivation of bacteria (Equation 4 and 5; Sakiyama et al. 2009; Estrela et al., 2002).

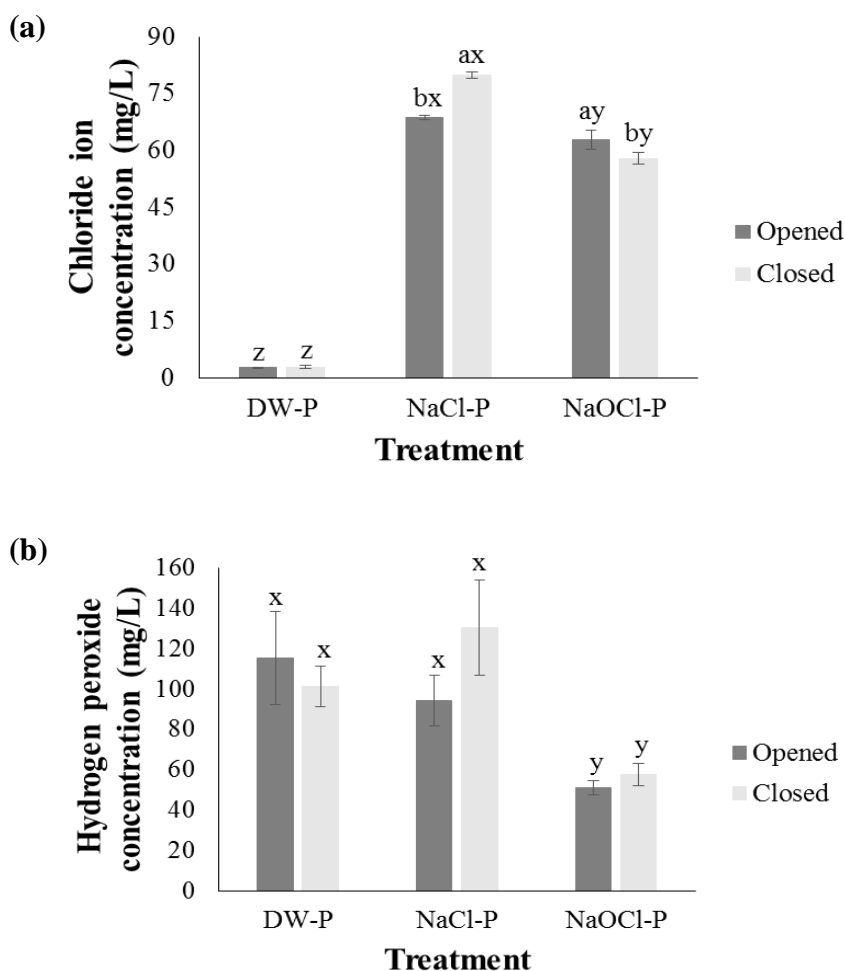


The generation of chemical species in the electrolyzed solution can decrease the pH value and increase the ORP, which related to the strong inactivation capability (Kiura et al., 2002). Chlorine species can rupture the cell wall and membrane and attack the bacteria not only from outside the bacteria but also from inside the cell, thus, significantly enhance the inactivation rate of bacteria (Fukuzaki, 2006). Chlorine as strong oxidant inhibits the enzymes reaction of bacteria through an irreversible oxidation of SH groups of critical bacterial enzymes (Estrela et al., 2002). Therefore, it was expected that NaCl-P and NaOCl-P that includes chlorine species was higher in reduction number of bacteria in biofilms than that of Direct-P and DW-P. However, the reduction number in NaCl-P was not significantly different between Direct-P and DW-P (Table 1).

Therefore, the concentration of chloride ions cannot explain the greatest bacterial reduction by NaOCl-P.

The concentration of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in NaOCl-P was 51.17 and 57.53 mg/L in opened and closed state, respectively (Fig. 8). Hydrogen peroxide functions as an oxidative stress to bacteria which can inactivate a variety of enzymes and rupture the cell membrane by cell lysis and lipid peroxidation (Imlay and Linn, 1988). Ikawa et al. (2010) confirmed that PTW included hydrogen peroxide can decrease the number of bacteria. Based on these previous results, present study conducted the microbiological analysis to investigate the inactivation of bacteria by hydrogen peroxide. Hydrogen peroxide can reduce the number of bacteria in the range of 2.3-2.8 log reduction at designated concentrations. The concentrations of hydrogen peroxide were prepared by the range generated in present plasma devices (Fig. 6). Therefore, it can be expected that DW-P and NaCl-P that includes higher hydrogen peroxide concentration compared to NaOCl-P were higher in reduction number of bacteria in biofilms. The lower concentration of hydrogen peroxide in NaOCl-P cannot explain the inactivation mechanism.

From the results so far, it can be thought that nitrite, nitrate, chloride ion, and hydrogen peroxide alone may not prove the inactivation efficiency of PTS against pathogenic bacteria in biofilms. There must be other chemical species that influenced the inactivation of biofilms on stainless steel NaOCl-P.

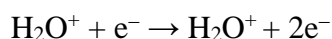
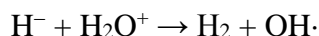
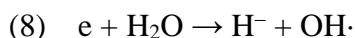
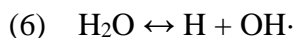


**Fig. 8.** Concentration (mg/L) of reactive species in plasma treated solution by different atmospheric pressure plasma methods. Abbreviation: DW-P, distilled water treated with plasma; NaCl-P, 100 ppm NaCl treated with plasma; NaOCl-P, 100 ppm NaOCl treated with plasma; Opened, the lid was opened immediately after plasma treatment; Closed, the lid was closed for 10 min after plasma treatment. (a) chloride ion, (b) hydrogen peroxide. <sup>a,b</sup>Different letters among the same solution differ significantly. <sup>x-z</sup>Different letter among the same post-duration treatments differ significantly ( $P<0.05$ ).

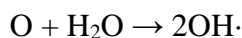
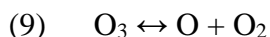
The concentration of hydroxyl radicals ( $\text{OH}\cdot$ ) in PTS was measured by the formation of phenol from benzene by using the reaction with hydroxyl radical and benzene, which can be converted into phenol. In closed state, the concentration of phenol in NaOCl-P was 0.34 mg/L which is higher than those of DW-P and NaCl-P. When compared between opened and closed state, the concentration of phenol in DW-P and NaOCl-P was higher in the closed state than that of the opened state, except for NaCl-P (Fig. 9).

Hydroxyl radicals ( $\text{OH}\cdot$ ) in PTS are known as highly reactive and have been contributed to inactivate the bacteria (Kim and Thayer, 1995). The formation of oxygen- and nitrogen-based reactive species by APP plays an important role in inactivation of microorganisms (Naïtali et al., 2010) as it may lead to important effects on the chemical reactions in the liquid phase and these radicals are precursors of other reactive species (Locke et al., 2006). Thus, the inactivation of biofilms in Direct-P treatment may be influenced by hydroxyl radicals and nitrogen-based reactive species. Electrical discharge could dissociate  $\text{H}_2\text{O}$  into hydroxyl radical (Equation 6; Locke and Shih, 2011). It was also suggested that hydrogen peroxide is dissociated by plasma treatment and result in decrease of hydrogen peroxide while increase of hydroxyl radical concentration (Equation 7). Equation 8 shows the recombination reaction to reform water from the radicals and reactive oxygen species production (Locke and Shih, 2011). Elevated intracellular levels of reactive oxygen species such as superoxide, hydrogen peroxide, and hydroxyl radicals can damage DNA, proteins, and lipids, consequently, oxidation stress results

in cell death (Imlay and Linn, 1988).

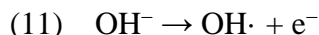
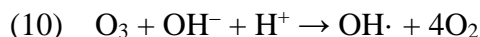


Generally, ozone was detected after plasma treatment and when submerged in water, ozone can live for 1000 sec in room temperature and results in reaction with water to produce hydroxyl radicals (Equation 9; Bai et al., 2011). Therefore, it was also suggested that generated ozone by plasma treatment reacts with water for a period time and produces hydroxyl radicals in the present study. Hydroxyl radicals are more powerful in inactivation of microorganisms due to its stronger oxidation potential (Sladek et al., 2007).

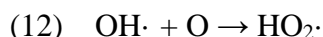


Further, the increase of hydroxyl radical concentration in NaOCl-P might be affected by dissociation of NaOCl into hydroxide ions ( $\text{OH}^-$ ; Equation 5). Hydroxide ions can be dissociated into hydroxyl radicals or

can produce hydroxyl radicals when react with O<sub>3</sub> in liquid phase (Equation 10 and 11; Attri et al., 2015).



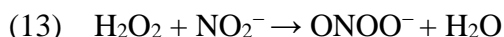
NaOCl solution, when in contact with organic tissue, releases hydroxyl radicals and forms chloramines which interfere cell metabolism (Estrela et al., 2002). When the 200 ppm NaOCl solution was treated to biofilms on stainless steel, the reduction number of *E. coli* O157:H7 and *L. monocytogenes* was 1.00 and 0.65 log CFU/cm<sup>2</sup>, respectively (data not shown). NaOCl-P resulted in higher bacterial reduction number in biofilms (Table 1) than that treated by 200 ppm NaOCl solution only. Therefore, it could be possible to decrease the amount of NaOCl use than typical practice when they are combined. Besides, through hydroxyl radicals production, relatively long-lived hydroperoxyl radical (HO<sub>2</sub>·) can be created (Equation 12) and inactivate the bacteria (Sakiyama et al., 2009).



Consequently, the combination of treated of plasma and NaOCl solution in closed state may induce synergistic effect on the inactivation of biofilms.

The concentration of peroxynitrite (ONOO<sup>-</sup>) was measured by NO<sub>2</sub>HPA which is a nitrated product of HPA. In closed state, the

concentration of NO<sub>2</sub>HPA in NaOCl-P was 0.48 mg/L and higher than that of DW-P and NaCl-P (Fig. 9). The reactions of nitrite with acidified hydrogen peroxide (Equation 13) and superoxide anion radicals (O<sub>2</sub><sup>·-</sup>) with nitric oxide (NO<sup>·</sup>) forms peroxynitrite (Equation 14; Saha et al., 1997).

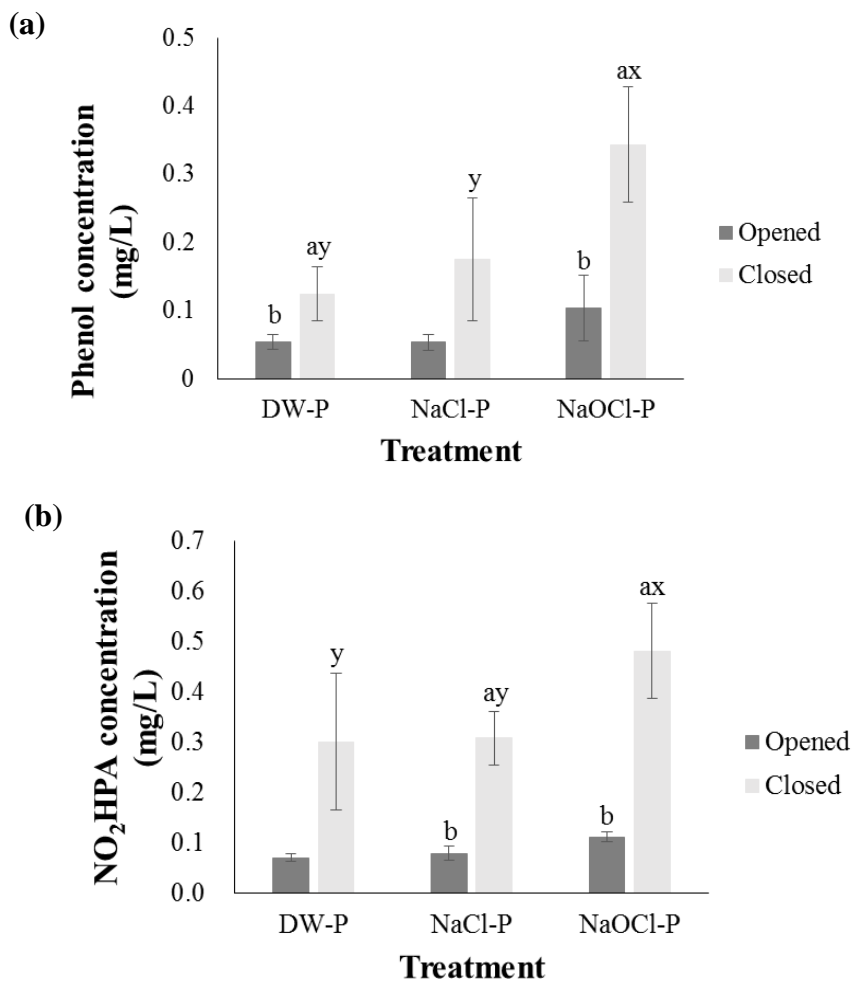


Present study shows that nitrite and hydrogen peroxide concentration in NaOCl-P were lower than that of other treatments (Fig. 8 and 9). The reduction of nitrite and hydrogen peroxide concentration might be occurred by the formation of peroxynitrite during post-duration time. The lower concentration of nitrite similar concentration of hydrogen peroxide in the opened state NaOCl-P explains the lower concentration of peroxynitrite in NaOCl-P when opened. In addition, it is possible that inactivation of bacteria in biofilms can be explained by peroxynitrite formed by the reaction of nitric oxide and superoxide anion radicals generated by the APP treatment (Ikawa et al., 2010). Naïtali et al. (2012) also reported that the oxidation reactions of peroxynitrite may occur at the liquid surface and in the aqueous phase. Peroxynitrite is strong oxidizer than hydrogen peroxide (Naïtali et al., 2012). Peroxynitrite is able to react with components of the bacterial membranes such as proteins, lipids, and lipoproteins that induce breakings of molecular bonding and results in inactivating the bacteria (Naïtali et al., 2012).



In addition, peroxynitrite is in equilibrium with peroxynitrous acid (ONOOH). Peroxynitrous acid can directly react with biomolecules in liquid phase (Szabó et al., 2007). Peroxynitrous acid dissociation to hydroxyl radicals ( $\text{OH}\cdot$ ) and nitrogen dioxide ( $\text{NO}_2\cdot$ ) seems to be relevant to initiate lipid peroxidation and nitration in membrane (Szabó et al., 2007).

From the results, the hydroxyl radicals and peroxynitrite produced by chemical reactions in NaOCl-P environment may play a major role in synergistic effect on the inactivation of *E. coli* O157:H7 biofilms.



**Fig. 9.** Concentration (mg/L) of reactive species in plasma treated solution by different atmospheric pressure plasma methods. Abbreviation: DW-P, distilled water treated with plasma; NaCl-P, 100 ppm NaCl treated with plasma; NaOCl-P, 100 ppm NaOCl treated with plasma; Opened, the lid was opened immediately after plasma treatment; Closed, the lid was closed for 10 min after plasma treatment. (a) phenol for hydroxyl radicals, (b) NO<sub>2</sub>HPA for peroxynitrite. <sup>a,b</sup>Different letters among the same solution differ significantly. <sup>x,y</sup>Different letter among the same post-duration treatments differ significantly ( $P < 0.05$ ).

## **4. Conclusion**

The synergistic effect in combination of plasma and NaOCl was confirmed against *E. coli* O157:H7 biofilms on stainless steel. The superior inactivation of this synergistic effect was due to the higher concentration of hydroxyl radicals and peroxynitrite that can be produced from the NaOCl-P environment. Using plasma technology, the lower concentration of NaOCl can be used than typical practice in industry.

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## V. Summary in Korean

최근 식품 안전성에 대한 관심이 높아짐에 따라 가공 공정에서 발생할 수 있는 미생물 오염을 줄이기 위해서 노력하고 있다. 하지만 이런 노력에도 불구하고 매년 수백만 건의 식중독이 발생한다. 가공 공정에서 많이 사용되는 기기나 파이프라인 재질인 스테인리스 스틸 표면에 형성된 바이오 필름이 식중독 원인 중 하나로 손꼽히고 있다. 일반적으로 이러한 바이오 필름을 제거하기 위해 차아염소산나트륨을 이용한다. 하지만 이를 이용하였을 때 독성 물질 발생 및 식품 표면 잔류에 대한 문제를 일으킬 수 있기 때문에 적절한 대체 방법으로서 비가열 살균 방법으로 주목 받고 있는 플라즈마를 이용하여 바이오 필름을 제어 하고자 한다. 또한 플라즈마 처리 후 밀폐된 공간에 방치할 경우 플라즈마 방전 이후에도 존재하는 화학종에 의해 살균 효과가 나타난다는 연구 결과가 있다. 뿐만 아니라 최근에는 플라즈마 처리수를 이용한 미생물 살균 효과 입증에 대한 연구가 주목 받고 있다. 하지만 이러한 플라즈마의 다양한 처리를 이용한 바이오 필름 제어에 대한 연구는 미미하다. 따라서 본 연구는 플라즈마의 다양한 처리를 통해 스테인리스 스틸에 형성된 바이오 필름 제어를 위한 최적 살균 조건을 찾고 바이오 필름에 대한 플라즈마 살균 기작을 연구하고자 진행하였다.

스테인리스 스틸에 형성된 *Escherichia coli* O157:H7 과 *Listeria monocytogenes* 바이오 필름에 대한 다양한 플라즈마 처리 방법에

대한 살균 효과를 확인하였다. *E. coli* O157:H7 에서는 차아염소산나트륨과 플라즈마를 함께 처리한 샘플에서 살균 효과가 가장 크게 나타났다. 하지만 *L. monocytogenes* 에서는 증류수와 플라즈마를 함께 처리한 샘플을 제외하고 플라즈마 처리 방법에 대한 살균 효과는 유의적 차이가 없는 것을 확인하였다. 이는 그람 양성균인 *L. monocytogenes* 가 가지고 있는 세포막 내 펩티도글리칸 층에 의해 그람 음성균인 *E. coli* O157:H7 에 비해 플라즈마에 대한 저항성이 상대적으로 크기 때문인 것으로 판단하였다.

바이오 필름에 대한 플라즈마 효과를 시각적으로 확인하기 위해 CLSM 과 SEM 을 촬영하였다. 대조군과 직접 처리, 그리고 차아염소산나트륨에 플라즈마 처리한 처리군을 비교하였을 때 대조군보다 각각의 처리군에서 바이오 필름 내 박테리아가 사멸한 것을 확인할 수 있었으며 플라즈마 의해 세포막 분리가 제대로 일어나지 않아 긴 막대 형태의 박테리아를 확인할 수 있었다.

플라즈마 처리수의 pH 와 ORP 측정한 결과 처리하지 않은 증류수에 비해 pH 는 감소하고 ORP 는 증가한 것을 확인하였다. 플라즈마 방전 시 화학종 발생 및 그로 인한 화학 반응으로 인해 처리수의 산성화가 일어나고 이는 박테리아 사멸에 효과적인 것으로 알려져 있으나 본 연구에서 pH 만으로 바이오 필름에 대한 미생물 제어 효과를 설명하기에는 부족하다. 플라즈마를 처리한 차아염소산나트륨 용액에서 ORP 값이 가장 높게 나왔다. ORP 값이 높다는 것은 그만큼 처리수 내 화학종이 많이 발생했음을 의미하고 본 실험에서 차아염소산나트륨에 플라즈마를 처리한 샘플에서 살균

효과가 가장 큰다는 결과를 뒷받침할 수 있을 것으로 판단된다. 또한 플라즈마 직접 처리시 OES 결과를 바탕으로 하이드록시 라디칼과 질소 화학종이 발생했음을 확인할 수 있었으며 이러한 화학종들은 용액 내에서 다양한 활성종의 전구체로 역할을 한다. 따라서 플라즈마 처리시 어떠한 화학종들이 얼마만큼 생성되었는지 확인하기 위한 실험을 진행하였다.

아질산염, 질산염, 과산화수소는 플라즈마 처리수 내에서 생성되어 처리수를 산성화시키며 미생물 제어에 효과적인 역할을 하는 것으로 알려져 있다. 하지만 플라즈마 처리 용액 내 아질산염, 질산염, 과산화수소 농도와 바이오 필름 살균 결과 상응하지 않으므로 단순히 이러한 화학종들만을 가지고 플라즈마의 살균 효과에 대해 설명하기 어렵다. 또한 염화나트륨 용액과 차아염소산나트륨 용액이 플라즈마에 의한 전해되었을 때 생성되는 염화 이온, 차아염소산 등에 의해 미생물 살균에 역할을 하는 것으로 알려져 있지만 본 실험에서 염화 이온 농도와 바이오 필름 살균 결과 상응하지 않으므로 단순히 염화 이온 농도만을 가지고 플라즈마 살균 효과에 대해 설명하기 어렵다. 하이드록시 라디칼은 차아염소산나트륨에 플라즈마를 처리하였을 때 농도가 높게 나타났다. 이는 용액 내 플라즈마에 의한 방전으로 발생하였을 뿐만 아니라 차아염소산나트륨이 물에 녹아 생성하는 수산화 이온에 의해 발생한 것으로 판단된다. 또한 과산화 질산염도 차아염소산나트륨에 플라즈마를 처리하였을 때 농도가 가장 높게 나타났다. 이는 아질산염과 과산화수소의 농도가 감소한 것을



근거로 과산화 질산염이 생성되었음을 확인할 수 있었다. 따라서 플라즈마와 차아염소산나트륨에 의한 하이드록시 라디칼과 과산화 질산염 생성이 바이오 필름 제어에 주된 역할을 한 것으로 판단된다.

결론적으로 스테인리스 스틸 표면에 형성된 바이오 필름 제어를 위해 차아염소산나트륨 용액(100 ppm)에 플라즈마를 처리하고 밀폐된 상태에서 보관하는 것이 가장 효과가 좋았으며 이는 플라즈마 처리수 내 존재하는 화학종인 하이드록시 라디칼과 과산화 질산염의 생성에 의한 것으로 사료된다. 본 연구 결과를 바탕으로 차아염소산나트륨 사용의 감소와 함께 가공 공정에서 식품의 안전성을 높이는데 플라즈마 기술이 적용될 수 있을 것으로 판단된다.